Regulation of arterial lesions in mice depends on differential smooth muscle cell migration: A role for sphingosine-1-phosphate receptors

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The response of mice arteries to injury varies significantly between strains. FVB mice develop large neointimas after injury, whereas very small lesions form in C57BL/6 mice. After injury, platelet interaction with the denuded artery and early smooth muscle (SMC) replication are identical in both strains; however, the migration of SMCs differs significantly. FVB cells readily move into the developing neointima, whereas only the occasional C57BL/6 cells migrate. Injured arteries showed no difference in matrix metalloproteinases (MMP-2 and MMP-9) and plasminogen activator activities. In vitro, sphingosine-1-phosphate (S1P) in combination with platelet-derived growth factor (PDGF) stimulates migration of FVB cells but inhibits migration of C57BL/6 SMCs. Both SMCs migrate equally well to PDGF alone. One explanation is that the SMCs express different S1P receptors. Real-time polymerase chain reaction shows that FVB cells express higher levels of S1P receptor-1 ($S1P_1$) compared with C57BL/6 cells, which express higher levels of S1P receptor-2 ($S1P_2$). In addition, the migration of C57BL/6 cells can be increased by inhibiting $S1P_2$, whereas inhibiting $S1P_1$ expression slows the migration of FVB cells. Taken together these studies suggest that expression of S1P receptors vary within inbred mouse strains and that S1P is critical for SMC migration and lesion formation after injury. (J Vasc Surg 2007;46:756-63.)

Clinical Relevance: **This report shows that the arteries of C57BL/6 and FVB mice vary in their ability to develop arterial neointimal lesions after injury. A major difference relates to ability of FVB smooth muscle cells to migrate into the intima and so develop neointimal lesions. In vitro FVB cells migrate well in response to sphingosine 1-phosphate (a component of plasma) but C57BL/6 cells do not. FVB cells express sphingosine-1-phosphate ($S1P_1$) and low levels of $S1P_2$ receptors, whereas C57BL/6 cells show higher $S1P_2$ expression. These data suggest that differences in S1P receptor expression are important role in the formation of neointimal lesions.**

Intimal thickening accompanied with lumen reduction plays a critical role both in arterial restenosis and atherosclerosis. Smooth muscle cells (SMCs) are a key component of these lesions, and as such, a multitude of studies have been done to understand how the proliferation of these cells is regulated. Much of our understanding on the growth of SMCs in vivo comes from studies in which arteries are deliberately injured and subsequently develop a thickened intima.

Harmon et al[1] first noted that the size of injury-induced intimal lesions in mice arteries was strain-dependent. The technique used in these studies to stimulate neointimal lesions was rather unusual and involved the complete ligation of the carotid artery. Why a neointima should form under these conditions is not understood, but other experiments, including our own in which a more traditional arterial injury was used, gave almost identical results.[2,3] It appears therefore that genetic variations significantly regulate neointimal formation in mice.

The aim of this study was to examine the arteries of two mice strains with very different responses to injury and resolve which cellular events, known to be critical for lesion growth, were impaired or different. The major finding of this study is that SMCs of C57BL/6 and FVB mice show a significant difference in their ability to migrate and we believe this is responsible for the difference in lesion size. We further show that differential expression of sphingosine 1-phosphate (S1P) receptors renders S1P promigratory in FVB SMCs and antimigratory in C57BL/6 SMCs. This difference might contribute to the strain-dependence of neointimal formation in injured mice arteries.

MATERIALS AND METHODS

Surgical procedure. Arteries in male mice (7 to 8 weeks old) were injured with a 6-0 monofilament catheter

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Competition of interest: none.

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introduced through the left external carotid artery and passed to the level of the carotid aortic bifurcation as previously described.[4] The catheter was then continuously rotated and slowly pulled back to the carotid bifurcation. This was repeated three more times. All studies were done within the guidelines for animal experimentation at the University of Washington.

In vivo proliferation assay and morphometry. Mice were injected intraperitoneally with bromodeoxyuridine (BrdU, 30 μg/g body weight) at 1, 9, and 17 hours before euthanasia. Carotid arteries were perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 minutes in situ. The arteries were then cut in two pieces at mid-length. Sections were prepared from the two segments starting at the cut end and then every 50 μm to obtain sections from six different sites (≥50 μm apart). Sections were then stained with hematoxylin and immunohistochemically stained with BrdU (Boehringer-Mannheim, Ingelheim, Germany) as previously described.[4]

Morphometric analysis of cross-sectional areas was performed on arterial sections by using computer-assisted image analysis (National Institutes of Health Image, Bethesda, Md). Hematoxylin-positive (total) and BrdU-positive (replicating) cells were counted on arterial sections.

Scanning electron microscopy. Mice arteries were perfused fixed with 4% paraformaldehyde and dehydrated tissue was examined by scanning electron microscopy (SEM). Ten images were taken of the artery at the same magnification, and platelet number was quantitated with a point-hit technique.

Zymography. Carotid arteries were processed as previously described.[4] Total protein (10 μg) was loaded onto 8% polyacrylamide gels containing 0.1% type I gelatin (Sigma, St Louis, Mo) for matrix metalloproteinase (MMP) activity and 0.1 % casein plus 14 μg/mL of plasminogen (Sigma) for plasminogen activity. After electrophoresis, the gels were incubated for 16 to 18 hours at 37°C and stained with Coomassie blue.

Mouse arterial smooth muscle cell isolation. Arterial SMCs were isolated from mouse carotid arteries by an enzyme dispersion approach using an enzyme mix of 2 mg/mL bovine serum albumin, 1 mg/mL collagenase, 0.375 mg/mL soybean trypsin inhibitor, and 0.125 mg/mL elastase type III in Hanks' balanced salt solution. After 10 minutes' incubation, the adventitial layer was removed, and the remaining tissue was incubated at 37°C for a further 2 hours. Cells were collected by centrifugation and placed in media containing 20% fetal bovine serum.

In vivo and in vitro migration assays. For in vivo migration, carotid arteries were perfusion-fixed at 7 days after injury as described previously and the vessels were opened longitudinally and pinned down onto agar plate with the luminal surface facing upward. Arteries were stained with hematoxylin. Migrated SMCs were identified as the hematoxylin-stained cells on the luminal surface of the endothelium-denuded area. The total number of migrated SMCs was counted.

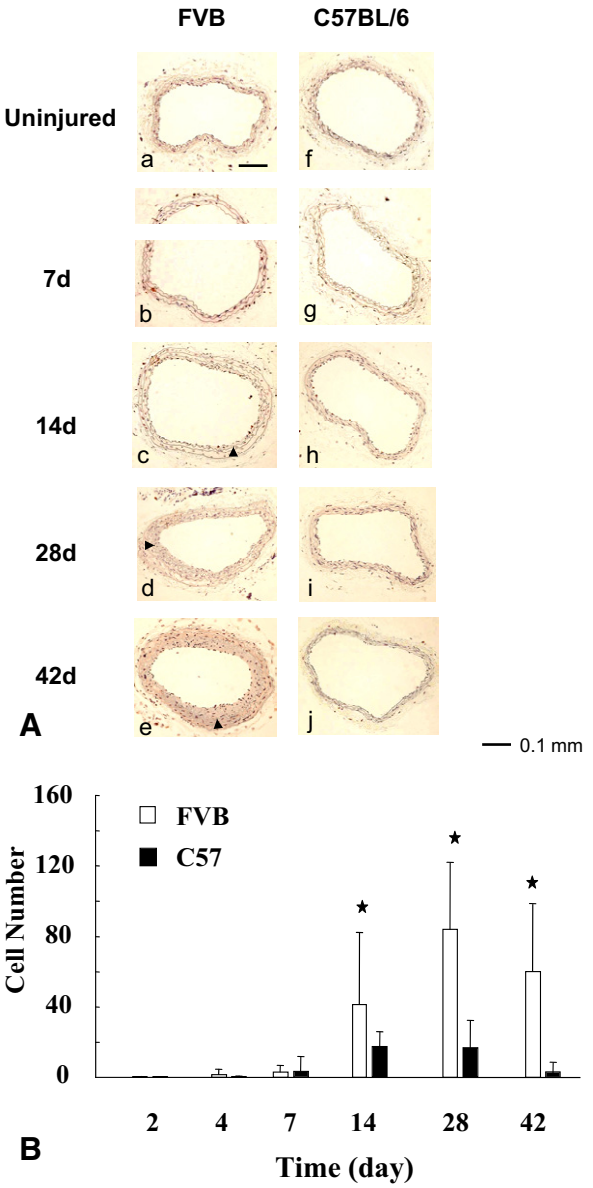

Fig 1. A, Neointimal lesions in FVB and C57BL/6 carotid arteries after injury. A small neointima can be seen *(c)* by 14 days, which *(d-e)* became significantly larger at 28 and 42 days in FVB arteries. Few neointimal cells can be seen *(f-j)* in C57BL/6 arteries at any time (n = 5 for each time point). The bar represents 0.1 mm. **B,** Quantification of intimal SMCs in injured FVB (clear bars) and C57BL/6 arteries (filled bars). At 14, 28, and 42 days, a significant increase in neointimal cell number was detected in FVB arteries. All values are standard deviations. *Significant at $P < 0.05$.

For in vitro migration, 1×10^5 cells were placed in the upper chamber of a 24-well Transwell (Costar, Corning Inc, Corning, NY) precoated with 0.1% type I collagen, and the medium containing 20 ng/mL platelet-derived growth factor (PDGF) or 1 μmol/L S1P, or both, was added to the lower chamber. After 7 hours, cells on the lower surface

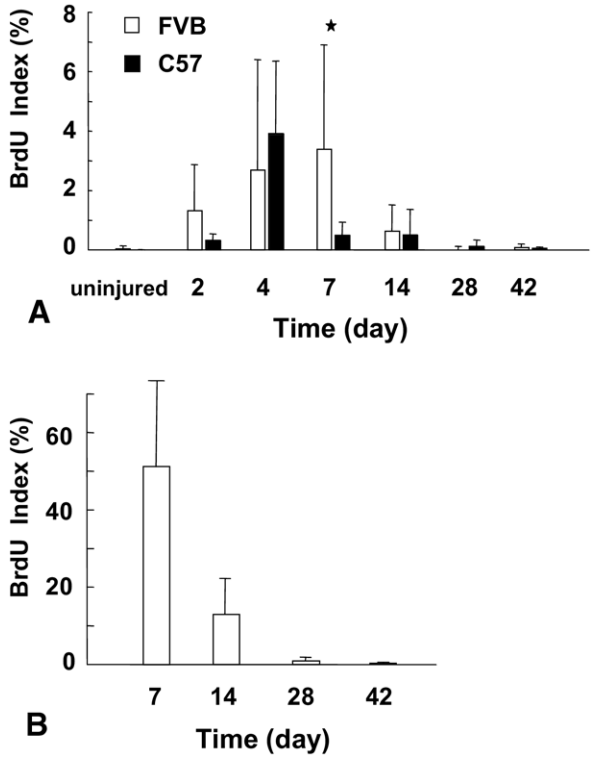

Fig 2. Smooth muscle cell (SMC) replication in FVB and C57BL/6 cells after injury. **A,** Medial SMC replication increased in both FVB (clear bars) and C57BL/6 arteries (filled bars) at 2 and 4 days after injury, but a significant difference in SMC replication between these arteries was observed only at 7 days. **B,** Intimal SMC replication was high after 7 days and to a lesser extent at 14 days. No increase was observed at 28 and 42 days. No replicating neointimal SMCs were detected in C57BL/6 arteries. All values are standard deviations. *Significant at $P < 0.05$. *BrdU,* Bromodeoxyuridine.

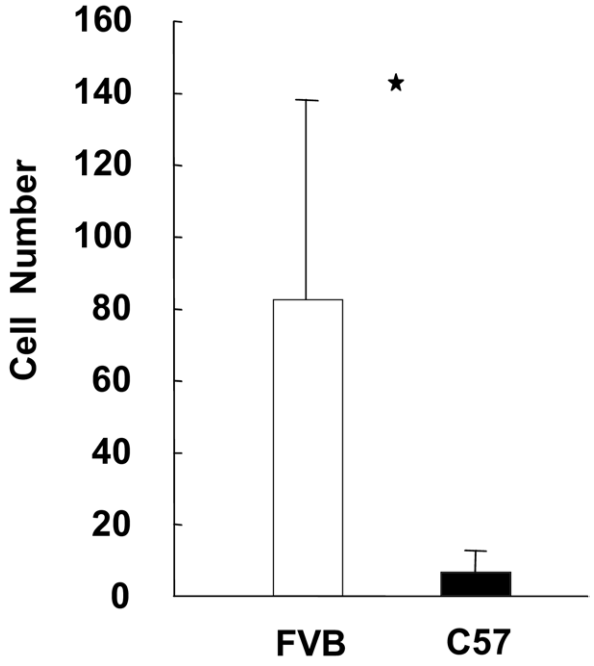

Fig 3. Quantitation of smooth muscle cell migration onto the luminal surface of injured FVB (clear bars) and C57BL/6 carotid arteries (filled bars). The number of lumenal cells was assessed along the entire length of the artery and expressed as cells/total area measured. All values are standard deviations. *$P < 0.05$ (11 mice/group).

were fixed with methanol and stained with hematoxylin. The cells on the lower surface of membranes (9 fields/membrane) were then counted under a microscope at ×40 magnification. The data are expressed as the mean number of cells/field.

Quantitative polymerase chain reaction. Complimentary DNA was prepared from each sample by treating RNA with DNase I (Promega, Madison, Wis) and reverse transcribed using Superscript reverse transcriptase (Promega). The expression of $S1P_1$ and $S1P_2$ was quantitated using a Taqman Sequence Detection Assay (PE Biosystems, Foster City, Calif). The fluorogenic probe, CGCCGCCTGACCTTCCGC was used for $S1P_1$ and TCCGCTCCAGCCACGCGG for $S1P_2$. Forward and reverse primers for $S1P_1$ were CTACTCCTTGGTCAGGACTCGAA and ACTCAGGACAATGATCACCGTCTT. For $S1P_2$ the forward primer was GTGGCTCTGTACGTCCGAATCTA, and the reverse primer CTAGCGTCTGAGGACCAGCAA. The same RNA samples were polymerase chain reaction (PCR) amplified for 18S and the S1P receptor expression was normalized to the 18S expression.

Primers for PAI-1 were 5′AGATGACCACAGCGGGGAAAAC and 3′AGAGGGCATTCACCAGCACCAG and for TIMP-1 were 5′GCATCTCTGGCATCTGGCATCC and 3′GCACACCCCACAGCCAGCACTA.

Small interfering RNA transfection. SMCs were transfected with $S1P_1$ and $S1P_2$-small interfering (si) RNA oligomers at 2 μmol/L concentrations (Invitrogen, Carlsbad, Calif) using an electroporation system (Amaxa Biosystems, Cologne, Germany). Cells were then allowed to recover for 24 hours in presence of 10% serum and siRNAs. Cells were serum-starved for additional 48 hours and then subjected to an in vitro migration assay as described above.

Statistical analysis. Differences between groups were evaluated by the unpaired Student *t* test. All data were considered significant at $P < .05$.

RESULTS

Response of FVB and C57BL/6 carotid arteries to denuding injury. Intimal SMCs were observed in FVB arteries at day 7 and their number and the size of the neointimal lesions increased with time up to 42 days (Fig 1). No significant lesions developed in C57BL/6 arteries. The size of the media measured by determining the area enclosed by internal and external elastic lamina showed no change after injury in either mice strain (data not shown).

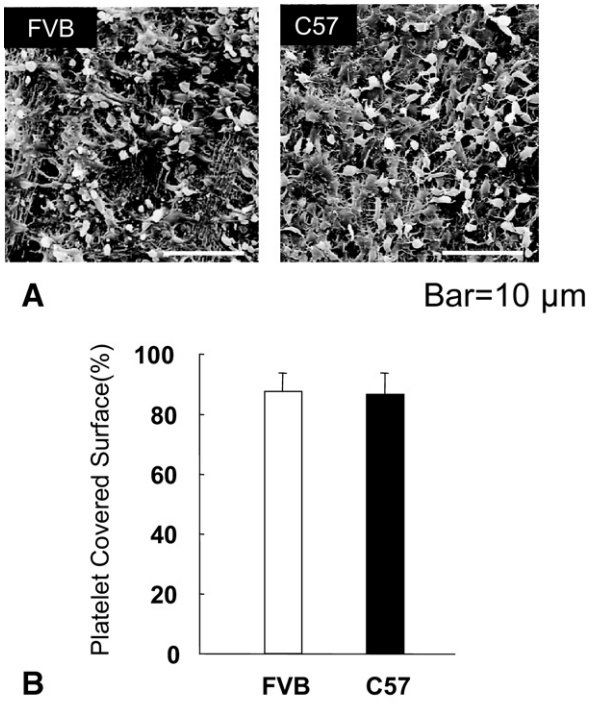

Fig 4. A, Scanning electron micrographs showing platelet adherence on the denuded surface of injured FVB and C57BL/6 carotid arteries 1 hour after denudation. Bar = 10 μm. **B,** The percentage of the denuded surface covered with platelets was quantitated from 15 micrographs for each artery (n = 5). All values are standard deviations.

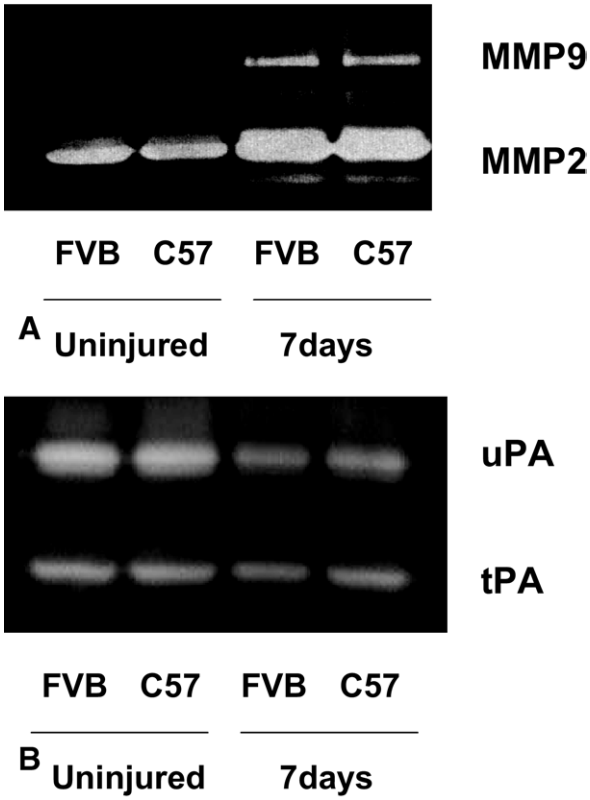

Fig 5. Activity of matrix metalloproteinases *(MMPs)* and plasminogen activators in injured FVB and C57BL/6 carotid arteries 7 days after injury. **A,** Zymogram containing gelatin shows MMP-9 and MMP-2 activities. **B,** Zymogram containing plasminogen and casein shows urokinase plasminogen activator *(uPA)* and tissue plasminogen activator *(tPA)* activities. The study was repeated three times and showed similar results.

At 2 and 4 days after injury, medial SMC replication was elevated in both FVB and C57BL/6 arteries compared with uninjured arteries; however, by day 7, replication was significantly higher in FVB arteries (Fig 2, *A*). Intimal cell replication was first detected in FVB arteries by day 7 and remained elevated at day 14; thereafter, cell replication decreased to almost undetectable levels (Fig 2, *B*). Owing to the infrequency of intimal SMCs in C57BL/6 arteries, no data are presented.

Migration of smooth muscle cells in injured FVB and C57BL/6 arteries. Migration of SMCs was evaluated by counting en face the number of SMCs on the entire luminal surface of the artery 7 days after injury. This time was chosen since SMCs are first observed on the luminal surface at this time.[4] As shown in Fig 3, there was significantly less migration in C57BL/6 arteries compared with the FVB mice at day 7.

We have previously reported that inhibition of SMC migration to the intima profoundly limits the size of the developing neointimal lesion and so we next investigated those events known to regulate SMC migration in an injured artery. The absence of platelets adhering to exposed subendothelium, such as in a thrombocytopenic animal, can significantly block SMC migration[5]; however, a normal platelet response was noted in both strains (Fig 4).

Several studies, including our own, have shown that MMPs and plasmin play an important role in SMC migration in injured arteries.[4] We therefore examined the activities of MMP-9, MMP-2, urokinase plasminogen activator (uPA), and tissue plasminogen activator (tPA) at the time when cells are known to be migrating from the media onto the luminal surface. No difference was observed in MMP-9 and MMP-2 or in uPA and in tPA activities as assessed by zymography between FVB and C57BL/6 injured arteries at the time when migration is known to occur (Fig 5, *A* and *B*). The expression of TIMP-1 and PAI-1 was similar for both sets of arteries 7 days after injury (data not shown).

Sphingosine 1-phosphate and smooth muscle cell migration. Recently S1P, a sphingolipid metabolite, has been found to influence SMC migration. S1P caused a dose-dependent increase in migration of FVB but decreased migration of C57BL/6 cells (Fig 6, *A*). Also, S1P in combination with PDGF increased FVB migration but exerted the opposite effect and inhibited PDGF-induced mi-

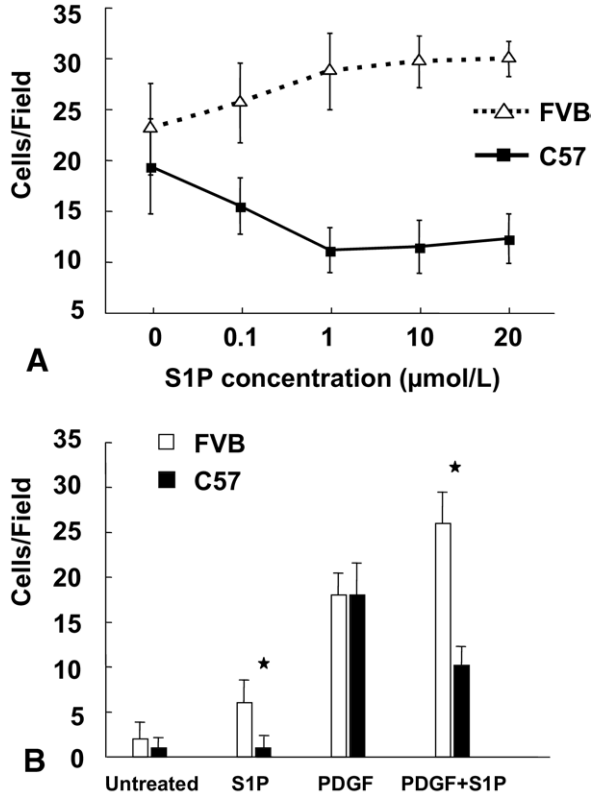

Fig 6. In vitro migration of FVB and C57BL/6 smooth muscle cells (SMCs). **A,** Migration of SMCs in response to 0, 0.1, 1, 10 and 20 μmol/L of sphingosine-1-phosphate *(S1P)*. Dotted line, FVB; solid line, C57BL/6. **B,** Cells were stimulated with 1 μmol/L S1P, 20 ng/mL platelet-derived growth factor-BB *(PDGF)*, and S1P and PDGF together (B). Each experiment was performed in triplicate and repeated three times. $^{*}P < 0.05$. All values are standard deviations.

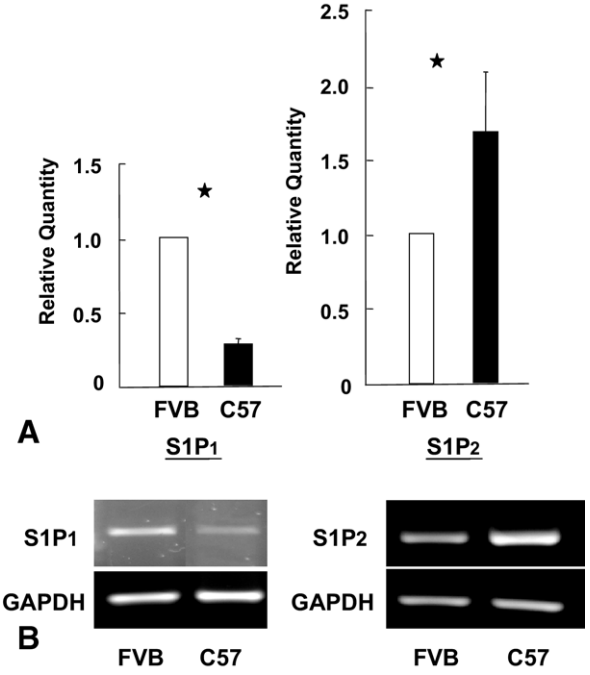

Fig 7. A, Real-time polymerase chain reaction (RT-PCR) shows expression of sphingosine-1-phosphate (S1P) receptors 1 *($S1P_1$)* and 2 *($S1P_2$)* in FVB and C57BL/6 smooth muscle cells. The data are expressed as the relative quantification of S1P receptors expression in C57BL/6 relative to the amount in FVB cells, which is set to one, and are presented with the standard error. Each experiment was repeated three times. $^{*}P < .05$. **B,** RT-PCR products of same samples (25 cycles). *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase.

gration of C57BL/6 cells (Fig 6, *B*). The response to PDGF alone was similar for both cells, suggesting no difference in PDGF receptor expression (Fig 6, *B*).

Sphingosine 1-phosphate receptor expression. SMCs express several S1P receptors and their activation can initiate different outcomes.[6,7] We therefore asked if differences in migratory ability of FVB and C57BL/6 cells is linked to expression of specific S1P receptors. Using real-time PCR we found that $S1P_1$ expression was stronger in FVB compared with C57BL/6 SMCs. In contrast, the $S1P_2$ receptor was expressed at higher level in C57BL/6 cells (Fig 7).

We then determined if down-regulation of S1P receptors by siRNA influences SMC migration. Inhibition of $S1P_1$ expression in FVB cells reduced their ability to migrate in response to S1P (Fig 8, *A* and *B*). Migration in response to PDGF was not changed. Treatment of C57BL/6 cells with the same siRNA also reduced $S1P_1$ expression but had no effect on migration in response to S1P, PDGF, and a combination of both factors (Fig 8, *A* and *C*). Inhibition of $S1P_2$ expression in FVB and C57BL/6 cells (Fig 9, *A*) promoted the migration of these cells in response to S1P and to a combination of PDGF and S1P (Fig 9, *B* and *C*). We therefore conclude that $S1P_2$ is the dominant receptor in C57BL/6 cells and retards SMC migration, whereas $S1P_1$, the dominant receptor in FVB, SMCs stimulates migration.

DISCUSSION

Response of FVB and C57BL/6 arteries to injury. In this study, we chose to examine C57BL/6 and FVB mice because there are data showing differences in the response of arteries to injury. Indeed, several laboratories, including ours, show that C57BL/6 arteries develop only small or no neointimal lesions after injury whereas FVB arteries form large neointimal lesions.[1-3,8] This finding is somewhat controversial because other laboratories show significant neointimal lesions in C57BL/6 arteries.[9-12]

It is difficult to resolve this disparity, although one explanation may be found in the way that arteries are injured. Unfortunately, there is no standard technique to denude or injure mice arteries and most laboratories manufacture their own catheters. These vary from metal guidewires and catheters with a variety of modifications to external cuffing of the artery as well as complete or partial

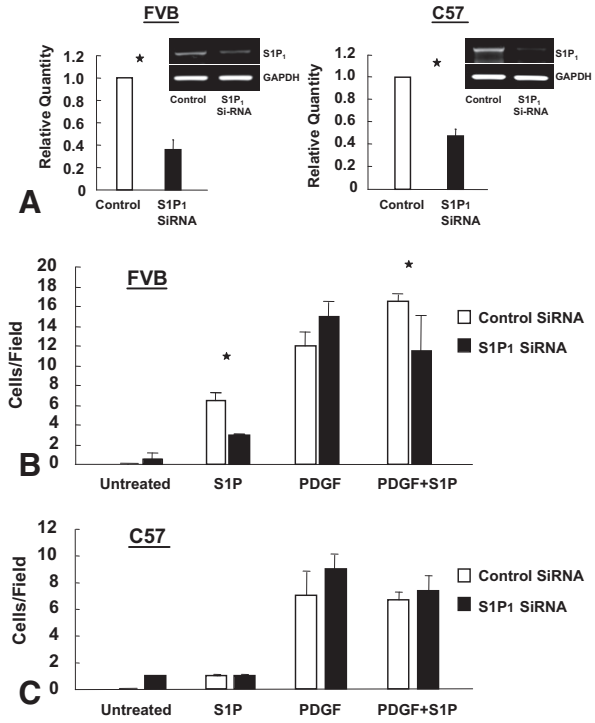

Fig 8. Migration of smooth muscle cells transfected with small interfering RNA (*siRNA*) targeting sphingosine-1-phosphate *(S1P)* receptor 1 *($S1P_1$)*. **A,** Real-time polymerase chain reaction shows $S1P_1$ receptor expression is down-regulated in both FVB and C57BL/6 cells with siRNA targeted to $S1P_1$ (2 μmol/L) and control siRNA (2 μmol/L). *Insert* shows RT-PCR products. Both **(B)** FVB and **(C)** C57BL/6 were then stimulated with 1 μmol/L S1P, 20 ng/mL platelet-derived growth factor *(PDGF)*, and a combination of both factors for both cells. Migration was determined after 7 hours (n = 3). All values are standard deviations. $^{*}P < 0.05$.

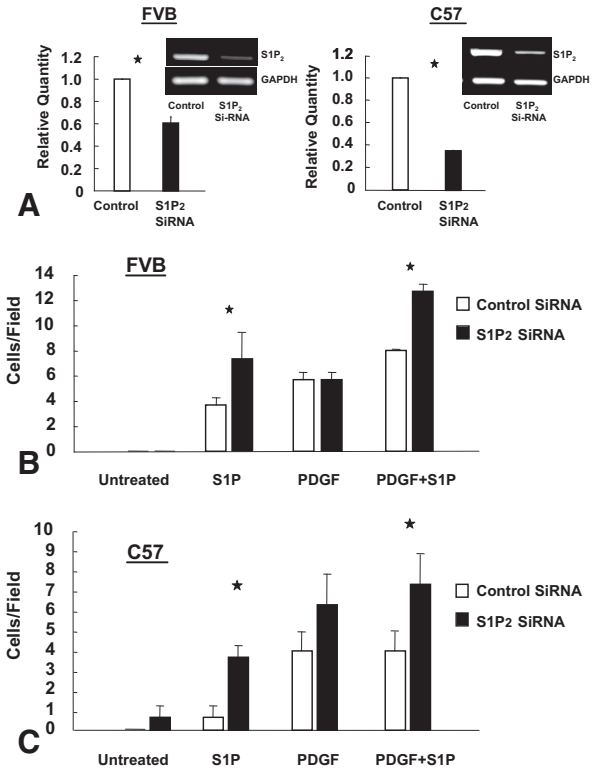

Fig 9. Migration of smooth muscle cells transfected with small interfering RNA *(siRNA)* targeting 2 μmol/L sphingosine-1-phosphate *(S1P)* receptor 2 *($S1P_2$)* and 2 μmol/L control siRNA. **A,** Real-time polymerase chain reaction (RT-PCR) results show that $S1P_2$ expression is downregulated in both cells. *Insert* shows RT-PCR product of same samples. The **(B)** FVB (B) and **(C)** C57BL/6 cells were then stimulated with 1 μmol/L S1P, 20 ng/mL platelet-derived growth factor (PDGF) and a combination of both factors for both cells. Migration was determined after 7 hours; n = 3. All values are standard deviations. $^{*}P < 0.05$.

restriction of blood flow.[3,13-15] With denuding catheters, the degree of trauma inflicted on the artery will likely vary, and authors have commented that lesions form at sites where the internal elastic lamella is ruptured, a phenomenon that we also observed.[16]

In our study, arterial injury was inflicted by passing a loop of 6-0 monofilament into the carotid artery. This method is similar to one we described for use in the rat.[15] An advantage of this procedure is that over-distension of the arterial wall is limited due to size and pliability of the filament and rupture of the elastic lamella does not occur. Steel guidewires and rigid catheters have a greater likelihood of inflicting severe trauma to the artery. Several techniques commonly used to elicit neointimal lesions in mice arteries, such as external cuffing or blood flow restriction, do not denude the artery, and so the events that propagate neointimal lesions are likely to be different from experiments that induce endothelial denudation and platelet interaction with the luminal surface.

This disparity in the response of mice arteries makes the interpretation of studies difficult. The results are consistent within each laboratory, yet important differences must have occurred although not currently recognized. With our procedure, we find consistently that C57BL/6 arteries develop small or no lesions, whereas FVB arteries invariably show significant neointimal lesions. This has held true for >200 mice of each strain.

FVB smooth muscle cells migrate well, but C57BL/6 cells do not. To understand why there are such marked differences between the arteries of these mice strains, we examined those cellular events thought to be necessary in the formation of neointimal lesions. The selection of the events to be studied was based on our extensive data derived from injured rat arteries and includes loss of endothelium, adherence of platelets, and SMC migration and replication.[17,18]

The first events known to have a major impact on the development of arterial lesions are loss of endothelium and the adherence of platelets. Platelets are known to regulate SMC migration, and this was thought to be due to the release of PDGF.[5,19,20] In both sets of arteries, the injury

totally denuded the endothelium and the number of platelets adhering to the injured surface was identical. Injury also triggers medial SMC replication, possibly by releasing fibroblast growth factor 2 from the traumatized SMCs.[19] Of interest is that both C57BL/6 and FVB arteries show a similar increase in medial replication at 2 and 4 days after injury. This last fact is significant because it suggests that there are no differences in the availability of growth factors or growth inhibitors for SMCs early after injury. The difference between these two strains presumably lies with events beyond this time period.

The first significant difference between the injured arteries of the two strains was noted at day 7, where migration of SMCs into the neointima was found to be significantly greater in FVB than in C57BL/6 arteries. Migration of SMCs is a critical event for the formation of a neointima in mice because these arteries do not normally possess an intima with SMCs and, consequently, cells must migrate from the media to the intima for neointimal growth to occur. Indeed, several studies have recently shown that if migration is blocked, then the formation of intimal lesions is completely retarded.[5,21-23] Proteolytic enzymes involved in this process include the plasminogen activators, tPA and uPA, as well as MMP-9 and MMP-2. One possibility, therefore, is that the mice strains used in this study vary in the expression of MMPs or proteases and this might explain why migration and the ultimate size of the lesions are so different. Our data show that this hypothesis is unlikely to be true because the activity of MMP-9 and MMP-2 and of the plasminogen activators uPA and tPA are similar at those times when migration is known to occur and no changes were found in TIMP-1 or PAI-1 expression.

In recent years, the sphingolipid S1P has been identified as a key regulator of cell motility.[24-29] S1P stimulates migration of several cells, yet interestingly, it inhibits SMC migration.[6,7,30-32] One reason why S1P might be important for the development of neointimal lesions is that S1P is released from platelets and so will be present at sites of arterial injury.[33,34] Further, a recent report showed that S1P levels in humans are predictive of future vascular events.[35] Because S1P can regulate SMC motility, we therefore asked if cells of these two mice strains respond differently to S1P. Our data show that S1P stimulated migration of FVB SMCs and, in combination with PDGF, the effect was additive. In contrast, S1P did not stimulate C57BL/6 cells and reduced the ability of cells to migrate towards PDGF. Thus, S1P modulates the migration of SMCs from C57BL/6 and FVB arteries, but in an opposite manner.

We next asked how S1P might regulate such different responses in SMCs from C57BL/6 and FVB strains. Because there are reports that differences in S1P receptor expression can influence SMC motility in opposite ways, we examined S1P receptor expression in C57BL/6 and FVB SMCs.[7,32,33] There are five known S1P receptors, $S1P_{1\text{-}5}$ although only $S1P_1$, $S1P_2$, and $S1P_3$ are expressed by SMCs.[35] The deletion of $S1P_1$ is lethal, and these mice die in utero because of incomplete vascular maturation.[36] In particular, there is a defect in the recruitment of SMCs and pericytes to the developing blood vessels. The expression of $S1P_2$, however, is linked to inhibition of migration, and this receptor is thought to be the dominant S1P receptor expressed by many SMCs.[32] C57BL/6 cells would fit into this category because they strongly express $S1P_2$, and more important, S1P blocks their migration. FVB SMCs, however, strongly express $S1P_1$ and less $S1P_2$, and their migration is increased by S1P. FVB SMCs, therefore, differ from the reported response of most SMCs.

To validate our hypothesis that differences in S1P receptor expression can account for the differences in SMC migration, we altered their expression. $S1P_1$ and $S1P_2$ expression was inhibited in C57BL/6 and FVB using siRNA targeted to each S1P receptor. A reduction of $S1P_2$ in C57BL/6 cells now promoted cell migration in response to S1P alone and combined with PDGF. A similar treatment also increased the migration of FVB cells. Targeting $S1P_1$ inhibited FVB SMC migration but had no effect on C57BL/6 SMCs. These data suggest that it is not the absolute level of S1P receptor but rather the balance of expression of the two receptors that decide if SMC migration is inhibited or promoted.

CONCLUSION

We have confirmed that injury to carotid arteries of FVB and C57BL/6 triggers different arterial responses reflecting a large difference in neointimal growth. The critical difference between these strains is related to the ability of SMCs to migrate to the intima. This migratory phenotype is maintained in vitro when cells are stimulated with S1P. We further demonstrate that different expression of $S1P_1$ and $S1P_2$ respectively regulate this phenotype. This work provides a basis to explain the difference between the arteries of these two strains and suggests an important role of S1P receptors expression in the development of arterial lesions.

AUTHOR CONTRIBUTIONS

Conception and design: MR, GD
Analysis and interpretation: MR, GD
Data collection: SI, TN, FD, DS
Writing the article: MR, AC, GD
Critical revision of the article: MR, AC, GD
Final approval of the article: MR, GD
Statistical analysis: AC
Obtained funding: MR
Overall responsibility: MR
SI and TN contributed equally to this work.

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